REMARKS

Claims 1, 5-10, 13-22, and 25-27 are pending. Claims 2 and 3 presently stand withdrawn as being drawn to non-elected subject matter. Applicants have cancelled claims 20, 26, and 27 without prejudice and added new claim 28. Claims 1, 5-10, 13-19, 21, 22, 25, and 28 will therefore be pending upon entry of the proposed amendments.

Applicants have deleted "halogen" from the listing of "Group B" substituents in claim 1. Support for this amendment can be found throughout the Specification, e.g., at page 13, line 20 through page 16, line 13.

Applicants have incorporated the limitations of claim 26 into claim 15. As such, claim 15 as currently amended is directed to a method for the treatment of "Alzheimer's disease" instead of a method for the treatment of "a memory disorder."

Applicants have incorporated the limitations of claim 27 into claim 16. As such, claim 16 as currently amended is directed to a method for the treatment of "depression" instead of a method for the treatment of "a mood disorder."

Applicants have deleted "sexual dysfunctions" and "urinary disorders" from the listing of disorders in claim 18. As such, claim 18 as currently amended is directed to a method for the treatment of epilepsy.

Applicants have amended claim 22 to further clarify the nature of the reactants used in the claimed method. Support for these amendments can be found throughout the Specification, e.g., at page 17, line 14 though page 22, line 10; and in the Examples delineated at page 24, line 24 through page 83, line 4. These amendments are discussed in more detail below.

Support for new claim 28 can be found throughout the Specification, e.g., at page 16, line 25.

No new matter is introduced by these amendments.

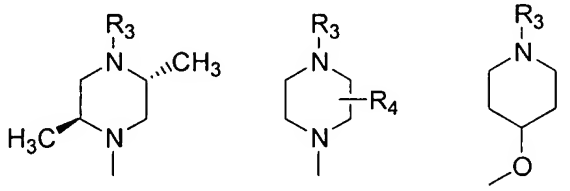
Rejection under 35 U.S.C. § 112, second paragraph

Claim 22 remains rejected under 35 U.S.C. § 112, second paragraph for allegedly "omitting essential steps" (Office Action, page 2).

The rejection has been met, in part, by amending the claims.

[1] Claim 22 is directed to a method for making various compounds of formula (I), in which one of R^1 and R^2 is a "Group A" substituent, and the other is a "Group B" substituent.

The Group A substituents include:



The "Group B" substituent include various oxygen, sulfur, and nitrogen-terminated substituents as well as aryl and heteroaryl rings. For purposes of discussion only, these various types of Group B substituents are each presented and discussed separately.

(i) aryl-C_1-C_6-alkoxy, heteroaryl-C_1-C_6-alkoxy, aryloxy-C_2-C_6-alkoxy, heteroaryloxy-C_2-C_6-alkoxy, 1-indanyloxy, 2-indanyloxy, aryloxy, heteroaryloxy, C_5-C_8-alkoxy, C_3-C_6-alkynyloxy, C_3-C_6-alkenyloxy, fluoro-C_2-C_4-alkoxy, C_4-C_8-cycloalkyloxy, C_3-C_8-cycloalkyl-C_1-C_4-alkoxy;

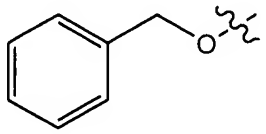
(ii) arylthio, heteroarylthio, C_5-C_6-cycloalkylthio, C_5-C_8-alkylthio, aryl-C_1-C_4-alkylthio, heteroaryl-C_1-C_4-alkylthio;

(iii) aryl-C_1-C_4-alkylamino, heteroaryl-C_1-C_4-alkylamino; or

(iv) heteroaryl, aryl.

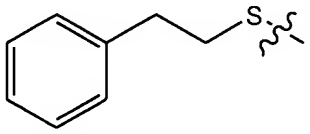
[2] Each of the Group A substituents is a cyclic amine that is attached to the pyrimidine ring of formula (I) *via* a ring nitrogen atom or an exocyclic oxygen atom.

Each of the Group B(i) substituents has the general formula, R-O-, and each is attached to the pyrimidine ring of formula (I) *via* the oxygen (O) atom. A non-limiting example of a Group B(i) substituent is a benzyloxy group (see Specification at page 37, Example 20):



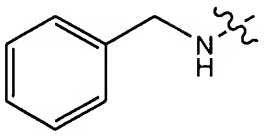
A benzyloxy group is a non-limiting example of an "aryl-C_1-C_6-alkoxy" substituent in claim 22.

Each of the Group B(ii) substituents has the general formula, R-S-, and each is attached to the pyrimidine ring of formula (I) *via* the sulfur (S) atom. A non-limiting example of a Group B(i) substituent is a 2-phenylethylsulfanyl group (see Specification at page 37, Example 20):



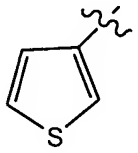
A 2-phenylethylsulfanyl group is a non-limiting example of an "aryl-C_1-C_4-alkylthio" substituent in claim 22.

Each of the Group B(iii) substituents has the general formula, R-NH-, and each is attached to the pyrimidine ring of formula (I) *via* the nitrogen (N) atom. A non-limiting example of a Group B(iii) substituent is a benzylamino group (see Specification at page 68, Example 82):



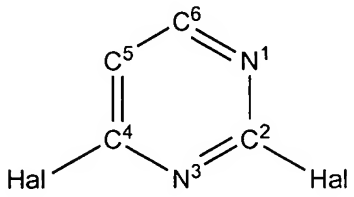
A benzylamino group is a non-limiting example of an "aryl-C_1-C_4-alkylamino" substituent in claim 22.

Each of the Group B(iv) substituents is an aryl or heteroaryl ring that is directly attached to the pyrimidine ring of formula (I) *via* a ring carbon. A non-limiting example of a Group (iv) substituent is 3-thienyl group (see Specification at page 34, Example 16):



A 3-thienyl group is a non-limiting example of an "heteroaryl" substituent in claim 22.

[3] The Specification teaches that the claimed compounds (as well as other compounds embraced by claim 1 as originally filed) can be prepared from a 2,4-dihalopyrimidine starting material of formula (**B**) (see Specification, e.g., at page 16, line 16 through page 21, line 15):



(**B**)

Each of the pyrimidine ring atoms in formula (**B**) is numbered according to convention, and each "Hal" in formula (**B**) represents a halogen.

The two halogens in formula (B) can be substituted, in a stepwise manner, by one group A substituent and by one group B substituent (or *vice versa*). Typically, the first substitution occurs at C-4 of the pyrimidine ring, and the second substitution occurs at C-2 of the pyrimidine ring.

More specifically, the Specification provides (page 17, line 19 through page 18, line 3, bold emphasis added):

> **Compounds of formula (I) above in which R_1 (or R_2) are bound to the pyrazine-, pyridine- or pyrimidine ring in (I) via an O, S or N** atom in R_1 (or R_2), are prepared by reacting a compound of the structural formula (III), (IV), (V), or (VI) .... *[halo substituted pyrazine-, pyridine- or pyrimidine rings omitted for clarity]* ... wherein Hal is halogen, **with an appropriate amine, alcohol or thiol** or its corresponding anion to produce a compound of formula (VII), (VIII), (IX), or (X) ...

In addition, the Specification teaches (page 21, lines 12-14):

> [T]he compounds of formula (I) wherein **R_1 or R_2 are aryl or heteroaryl** may be prepared by reacting a compound of formula (III), (IV), (V), or (VI) with a **boronic acid derivative of the type heteroaryl-$B(OH)_2$ or aryl-$B(OH)_2$**, ...

By way of example, a person of ordinary skill in the art would understand that a compound of formula (I) in which R^1 or R^2 is benzyl**oxy**, 2-phenylethyl**sulfanyl**, or benzyl**amino** can be prepared by reacting the appropriate halo-pyrimidine with the corresponding **alcohol** (i.e., benzyl alcohol), **thiol** (i.e., 2-phenylethanethiol), or **amine** (i.e., benzylamine), respectively. Similarly, a person of ordinary skill in the art would understand that a compound of formula (I) in which R^1 or R^2 is 3-thienyl could be prepared by reacting the appropriate halo-pyrimidine with the corresponding **boronic acid** (i.e., thiophen-3-boronic acid). See, e.g., examples 16, 20, 82, and 118 cited above.

[4] Steps (a) and (b') in claim 22 as currently amended now recite "contacting" a 2,4-di-halopyrimidine (i.e., formula B) and 2-monohalopyrimidine (i.e., formula VIII), respectively, with an alcohol, thiol, amine, or boronic acid selected from the group consisting of:

(i) R-OH, wherein R is aryl-C_1-C_6-alkyl, heteroaryl-C_1-C_6-alkyl, aryloxy-C_2-C_6-alkyl, heteroaryloxy-C_2-C_6-alkyl, 1-indanyl, 2-indanyl, aryl, heteroaryl, C_5-C_8-alkyl, C_3-C_6-alkynyl, C_3-C_6-alkenyl, fluoro-C_2-C_4-alkyl, C_4-C_8-cycloalkyl, or C_3-C_8-cycloalkyl-C_1-C_4-alkyl, each of which is optionally substituted;

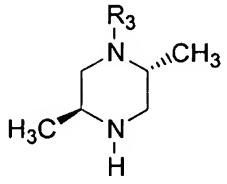
(ii) R-SH, wherein R is aryl, heteroaryl, C_5-C_6-cycloalkyl, C_5-C_8-alkyl, aryl-C_1-C_4-alkyl, or heteroaryl-C_1-C_4-alkyl, each of which is optionally substituted;

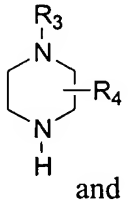
(iii) R-NH_2, wherein R is aryl-C_1-C_4-alkyl or an heteroaryl-C_1-C_4-alkyl, each of which is optionally substituted;

(iv) R-$B(OH)_2$; wherein R is heteroaryl or aryl, each of which is optionally substituted;

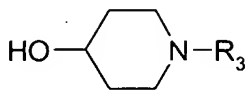
to form a mono- and di-substituted product, respectively (i.e., compounds of formula IX and I, respectively). In other words, claim 22 now recites the (generic) alcohol, thiol, amine, and boronic acid counterparts of the Group B substituents recited at the beginning of claim 22.

Likewise, steps (b) and (a') in claim 22 as currently amended now recite "contacting" a 2-monohalopyrimidine (i.e., formula IX) and a 2,4-di-halopyrimidine (i.e., formula B), respectively, with one of the following compounds:





and



(XI)

to form a di- and mono-substituted product, respectively (i.e., compounds of formula I and VIII, respectively). These three compounds are the amine and alcohol counterparts of the Group A substituents recited at the beginning of claim 22.

[5] Thus, claim 22 as currently amended includes a recitation of starting materials, intermediate compounds, and an end product and is therefore complete. In addition, the present claims interrelate the essential steps of the claimed methods. Each of the steps recited in claim 22 sets forth its own starting material and end product. Specifically, the product obtained in step (a) of claim 22 as currently amended serves as the starting material for step (b) in claim 22, and the product obtained in step (a') of claim 22 as currently amended serves as the starting material for step (b') in claim 22. Thus, in each step, it is self-evident, for example, which bonds are broken in the particular starting material and which formed in the end product. Further, it is also clear which atoms are introduced and which are eliminated in the starting material and end product of each step.

In view of the foregoing, Applicants submit that claim 22 does not omit matter disclosed to be essential to the claimed method, nor does the present claims fail to interrelate the essential elements of the claimed methods. Applicants respectfully request that the rejection be withdrawn.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 15, 16, 18, 20 and 26 were rejected under 35 U.S.C. §112, first paragraph, as not enabled. While the Examiner acknowledged that the specification enables the treatment of obesity, eating disorders, anxiety, depression, epilepsy, pain and schizophrenia, the Examiner argued that the specification does not enable treatment of all mood disorders, all memory disorders, all sexual dysfunctions, or all urinary disorders. The Examiner concludes by stating that there is "no adequate written description and enabling disclosure in the instant specification" for these disorders

Claim 15 has been amended to recite only treatment of Alzheimer's disease. Claim 16 has been amended to recite only treatment of depression, which the Examiner has acknowledged

is enabled, and claim 18 has been amended to recite only treatment of epilepsy which the Examiner has acknowledged is enabled. Claim 20 has been cancelled as have claims 26 and 27.

There is ample evidence that the 5-HT_{2c} receptor is a useful target for the treatment of Alzheimer's disease. For example, Arjona et al. (*Brain Research* 951:135, 2002; copy enclosed) examined the effect of dexnorfenfluramine (DEXNOR) on APP_S levels in the cerebrospinal fluid of guinea pigs. As Arjona et al. explain (page 136), guinea pigs were chosen as a model system because guinea pig and human APP have 98% sequence homology and because their Aβ peptide sequences are identical. Arjona et al. found that DEXNOR administration increases APP_S levels in the cerebrospinal fluid and, when administered chronically, decreases levels of $A\beta_{1-42}$. Importantly, Arjona et al. found that the increase in cerebrospinal fluid APP_S associated with DEXNOR was completely blocked by a 5-$HT_{2A/2C}$ antagonist, but <u>not</u> by a 5-HT_{2A} antagonist. Based, in part on this finding, Arjona et al. state that "in vivo, the main effect of DEXNOR is mediated via 5-HT_{2C} receptors." The authors conclude by stating that agonists of 5-HT2C receptors may be useful for treating Alzheimer's disease and may exhibit fewer side effects than other treatments have a similar effect on APP.

> Our data indicate that serotoninergic compound like DEXNOR that activate brain 5-HT2C receptors may be useful for treating AD by increasing APP_S formation and decreasing that of $A\beta_{1-42}$. Because 5-HT_{2C} receptors are apparently localized to the brain ... 5-HT_{2C} agonists may not produce peripheral side effects as seen with other drugs ... that similar non-amyloidogenic APP cleavage.

In view of the teachings of Arjona et al., it is evident that those skilled in the art find it credible that 5-HT_{2C} agonists are treatments for Alzheimer's disease. The present specification enables those skilled in the art to make and to use the compounds of claims 1, 5-10, and 25 for the treatment of Alzheimer's disease.

Applicants also respectfully request that the rejection not be applied to new claim 28, which is directed to the treatment of urinary incontinence. As discussed in the previous response, there is an established link between 5-HT_{2C} modulation and urinary incontinence.

In view of the forgoing, Applicants respectfully request that the rejections under 35 U.S.C. §112, first paragraph be withdrawn.

Rejection under 35 U.S.C. § 103(a)

Claims 1, 5-10, 13-22, and 25-27 remain rejected under 35 U.S.C. § 103(a) as being unpatentable over Baroni et al., EP 0 580 465 (Baroni). According to the Office (Office Action, page 8):

> [T]he definition of, X, Y, R, R_1 and R_2 overlap with the instant variable groups. Note when R_2 is halogen, the compounds taught by Baroni et al. include the instant compounds.

The rejection has been met by amending the claims. More specifically, Applicants have deleted "halogen" from the listing of "Group B" substituents in claim 1. Baroni does not teach or suggest compounds meeting the limitations of the present claims. In view of the foregoing, Applicants respectfully request that the rejection be withdrawn.

CONCLUSION

Applicants submit that all claims are in condition for allowance.

Enclosed is a $1,020 check for the Three Month Petition for Extension of Time fee. Please apply any other charges or credits to deposit account 06-1050, referencing Attorney Docket No.: 13425-056002.

Respectfully submitted,

Date: January 26, 2007

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Research report

Effect of a 5-HT_{2C} serotonin agonist, dexnorfenfluramine, on amyloid precursor protein metabolism in guinea pigs

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Accepted 21 June 2002

Abstract

Stimulation of serotonin receptor subtypes 5-HT_{2A} or 5-HT_{2C} in stably transfected 3T3 cells by dexnorfenfluramine (DEXNOR) or serotonin increases secretion of the APP metabolite APP_s. It is not known whether activation of these receptors can also affect APP metabolism in vivo. We examined the effects of a single intraperitoneal (i.p.) injection of DEXNOR on APP_s levels in cerebrospinal fluid (CSF) of guinea pigs. These levels were significantly ($P<0.05$) increased by a single dose of DEXNOR (1–4 mg/kg); those of the APP metabolites $A\beta_{1-40}$ and $A\beta_{1-42}$ were unaffected. The DEXNOR-induced (1 mg/kg) increases in CSF APP_s were suppressed by ritanserin (1 mg/kg) but not by ketanserin (2 mg/kg). When given alone, ritanserin did not affect CSF levels of APP_s, $A\beta_{1-40}$, or $A\beta_{1-42}$. Chronic treatment with DEXNOR for 9 days (1 mg/kg bid, i.p.) increased CSF APP_s levels, measured 2 h after the last injection ($P<0.05$), and decreased those of CSF $A\beta_{1-42}$ ($P<0.05$). Neither hippocampal nor cortical levels of the APP holoprotein (APP_h), nor body weight, were affected by DEXNOR. Chronic administration of mCPP (1-(*m*-chlorophenyl)piperazine) (2 mg/kg bid, i.p.), a 5-$HT_{2B/2C}$ agonist, for 9 days also increased CSF APP_s levels ($P<0.5$) when measured 2 h after the drug's last administration; hippocampal and cortical APP_h levels were unaffected. However, mCPP also caused a significant decrease in body weight gain. These data indicate that the pharmacological activation of 5-HT_{2C} receptors can stimulate CSF APP_s secretion and reduce Aβ production in vivo. Hence 5-HT_{2C} receptors, which apparently are localized to the brain, may represent useful targets for the development of treatments for Alzheimer's disease.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Serotonin receptors

Keywords: Dexnorfenfluramine; mCPP; Guinea pig; Alzheimer's disease; APP; Aβ; Serotonin agonist; Ritanserin; Ketanserin

1. Introduction

Therapeutic agents currently prescribed for Alzheimer's disease (AD) are cholinomimetic substances, i.e., acetylcholinesterase inhibitors. Although dysfunction of long-axon cholinergic brain neurons is an early manifestation of AD, attempts to slow the progression of AD with these drugs have had only modest success, perhaps because the doses that can be administered are limited by peripheral cholinergic side effects. In vitro activation of muscarinic m_1 or m_3 receptors in human embryonic kidney cells increases APP_s secretion [16] by releasing diacylglycerol (DAG) from membrane phosphatides, and concurrently decreases Aβ production [5]. In vivo, selective m_1 agonists have also been shown to decrease cerebrospinal fluid Aβ concentrations in rabbits and humans [1,18]. However, the muscarinic receptors exist in both the central nervous system and peripheral tissues, hence their activation can cause adverse peripheral side effects. Moreover, muscarinic agonists have not clearly been shown to improve cognition in AD [7,8].

Activation of 5-HT_{2A} or 5-HT_{2C} receptors in transfected 3T3 cells by serotonin or DEXNOR has been shown to increase APP_s secretion, also by liberating DAG [17]. We

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PII: S0006-8993(02)03153-0

have examined the effects of 5-HT-2 agonists in vivo by measuring APP_s (and, for DEXNOR, Aβ) in cerebrospinal fluid of guinea pigs treated with DEXNOR or mCPP. We chose this species because guinea pig and human APP exhibit 98% sequence homology [3], the proteins are processed similarly [2], and their Aβ peptide sequences are identical [6].

2. Materials and methods

2.1. Cerebrospinal fluid withdrawal

Male Dunkin Hartley guinea pigs (Charles River, Wilmington, MA, USA) (300 g, 28 days of age) were given access to feed (Prolab RMH 3000, PMI Nutrition International, St. Louis, MO, USA; 22% protein) and water ad libitum, and exposed to a 12:12 light:dark cycle. Animals were allowed to habituate to the environment for 1 week prior to being used in an experiment. All treatment groups contained six or more animals.

CSF collection was carried out in fully anesthetized animals (ketamine/xylazine, 40 and 5 mg/kg, respectively) as described by Kusumi and Plouffe [9] with minor modifications. Briefly, the cervical area was shaved and cleansed with alcohol. A 23 gauge needle without its hub was attached to a length of PE60 tubing (I.D. 0.76 mm, O.D. 1.22 mm; Becton Dickinson, Sparks, MD, USA) connected to a 1 cc syringe. The needle was then used to penetrate the cisterna magna at the atlanto–occipital joint. CSF (<100 μl) was withdrawn over 10 s. CSF was collected once per session, and no more than once per week. Samples contaminated with blood were discarded. The entire procedure was completed within 4 min.

2.2. Dose response studies

The effects on CSF APP_s, $Aβ_{1-40}$ and $Aβ_{1-42}$ levels of acute administration of varying concentrations of DEXNOR were determined as follows. Guinea pigs randomly assigned to each treatment group were injected (i.p.) with DEXNOR (0.5, 1.0, 2.0 or 4.0 mg/kg) or buffer. The DEXNOR was generously provided by Technology Servier (Paris, France) or synthesized by Professor Timothy Maher (Massachusetts College of Pharmacy, Boston, MA, USA). DEXNOR and all other compounds administered were dissolved in dimethyl sulfoxide (DMSO). CSF was collected after 2 h and processed as described below.

2.3. Time course studies

Guinea pigs randomly assigned to each treatment group received DEXNOR (1 mg/kg, i.p.) or DMSO. CSF was collected after 1, 2, 4 or 8 h.

2.4. Antagonists

To examine the effects of the serotonin antagonists ritanserin (1 mg/kg) and ketanserin (2 mg/kg) (RBI–Sigma, St. Louis, MO, USA) on DEXNOR-induced increases in CSF APP_s, animals were injected (i.p.) with one of the antagonists, or DMSO, 2 h prior to receiving DEXNOR (1 mg/kg, i.p.), and CSF samples were collected after 2 h.

2.5. Chronic administration studies

2.5.1. Dexnorfenfluramine

Animals received daily injections of DEXNOR (1 mg/kg bid, i.p.) for 9 consecutive days, and body weights were determined every 2 days. On the ninth day, some animals in the DEXNOR-treated groups received their last injection 2 h prior to CSF collection, while the remaining animals in the treated group received their last injection 24 h prior to CSF collection. After CSF withdrawal the guinea pigs were sacrificed, and the hippocampus and cortex were dissected and processed for analysis of APP_h.

2.5.2. mCPP

To assess the effects of chronic mCPP (Tocris, Ballwin, MO, USA) administration on APP metabolism and body weight, animals received mCPP (2 mg/kg bid, i.p.) for 9 consecutive days, and were weighed every 2 days. On the ninth treatment day, the mCPP group received its last injection 2 h prior to CSF collection for APP_s assay. After CSF withdrawal, the guinea pigs were sacrificed and the hippocampus and cortex were dissected and processed for APP_h analysis.

Guinea pigs were used repeatedly and were randomly assigned to the various treatment groups for the dose–response, time-course, and receptor–antagonist studies. A minimum of 1 week was allowed to pass between studies. Body weights were determined weekly for the duration of the studies unless otherwise stated. Naïve guinea pigs were used for studies involving chronic DEXNOR and mCPP administration.

2.6. Western blot analysis and ELISA

An aliquot of CSF was diluted 1:1 with 2× sample buffer (125 mM Tris, pH 6.8, 8% SDS, 40% glycerol, 10% β-mercaptoethanol, 1.4% bromophenol) prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis. APP_h concentrations were determined using approximately 10 mg of temporal cortex or hippocampal tissue. Each sample was placed in a microfuge tube containing 200 μl lysis buffer (60 mM Tris–HCl, 4%, 20% glycerol, 1 mM dithiothreitol, 1 mM AEBSF, 8 μM Aprotinin, 500 μM Bestatin, 15 μM E64, 200 μM Leupeptin, 10 μM Pepstatin A). The samples were then sonicated, boiled (10 min), and centrifuged (14,000×g/1 min/room tempera-

ture). The supernatant fluid was transferred to a clean microfuge tube and total protein concentrations in this fluid and in CSF samples were determined using the Bicinchoninic Acid (Sigma, St. Louis, MO, USA) assay.

Equal amounts of protein were loaded for electrophoretic analysis (4–20% gradient) (BioRad, Hercules, CA, USA). Proteins were then transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore) and incubated in 5% bovine serum albumin (Tris-buffered saline/0.15% Tween 20) for 1 h. Monoclonal antibodies 22C11 (Roche, Indianapolis, IN, USA) and APP-KPI (Chemicon, Temecula, CA, USA) were used to detect the N-terminus and KPI-domain of APP, respectively. In addition, an antibody against glial fibrillary acidic acid (GFAP) (Chemicon) was used. Protein–antibody complexes were detected and visualized using the ECL system (Amersham, Piscataway, NJ, USA) and Kodak X-AR film, respectively, as suggested by the manufacturer. Films were digitized using a Supervista S-12 scanner with a transparency adapter (UMAX Technologies, Freemont, CA, USA). Analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institute of Health) and available on the internet at http://rsb.info.NIH.gov/NIH-IMAGE/.

2.7. $A\beta_{1-40}$ and $A\beta_{1-42}$ measurements

CSF $A\beta_{1-40}$ or $A\beta_{1-42}$ levels were determined using commercially available ELISA kits for human Aβ (BioSource International, Camarillo, CA, USA). CSF samples were diluted 1:2 using sample diluent prior to analysis and processed as described in the manufacturer's instructions. Total protein in the CSF was determined using the Bicinchoninic Acid (Sigma) assay. Aβ levels were corrected for protein content.

2.8. Data analysis

Measurements of cellular and secreted proteins were normalized against those of control groups. Analysis of variance (ANOVA) was used to determine differences between groups (significance level, $P \leq 0.05$), using drug treatments as the independent variable. When differences were detected, the means were separated using Dunnett's test. Data are presented as mean±S.E.M.

3. Results

A single intraperitoneal injection of DEXNOR (1, 2 or 4 mg/kg) caused a significant increase in CSF APP_s levels (1.51±0.13-, 1.87±0.18- and 1.75±0.13-fold control values, respectively; $P<0.05$) of guinea pigs 2 h after its administration (Fig. 1). CSF $A\beta_{1-42}$ levels, however, were not significantly different from those of controls (Fig. 1).

Ritanserin (1 mg/kg) blocked the DEXNOR-induced

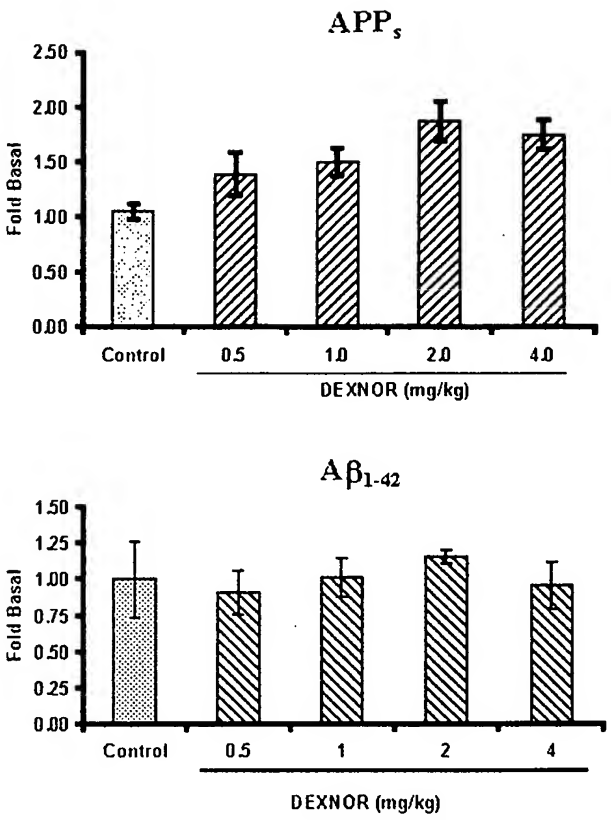

Fig. 1. DEXNOR-induced increases in CSF APP_s levels of guinea pigs. A single intraperitoneal injection of DEXNOR (1, 2 or 4 mg/kg) caused a significant (*$P<0.05$) increase in CSF APP_s levels 2 h after its administration. CSF $A\beta_{1-42}$ levels were not different from those of controls. Values represent mean±S.E.M.

(1.60±0.14) increase in CSF APP_s levels when compared with the increases seen in control animals or in guinea pigs treated with ritanserin alone (1.03±0.067). Ketanserin failed to do so (2 mg/kg) (1.33±0.193) ($P>0.05$) (Fig. 2). When administered alone, ritanserin did not have a significant effect on CSF APP_s levels (Fig. 2).

Time-course (1, 2, 4 and 8 h) studies showed that,

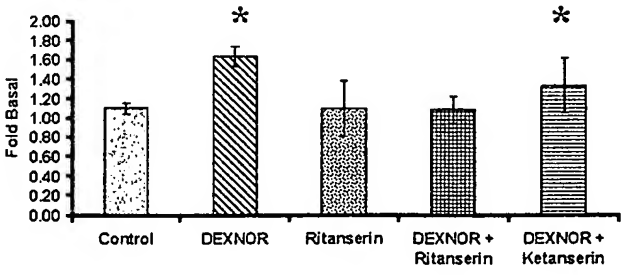

Fig. 2. Effect of the serotonin antagonists ritanserin and ketanserin on DEXNOR-induced increases in CSF APP_s levels. When compared to control, ritanserin (1 mg/kg) but not ketanserin (2 mg/kg) (*$P<0.05$) suppressed the DEXNOR-induced rise in CSF APP_s. When administered alone, ritanserin did not have an effect on APP_s CSF levels. Values represent mean±S.E.M.

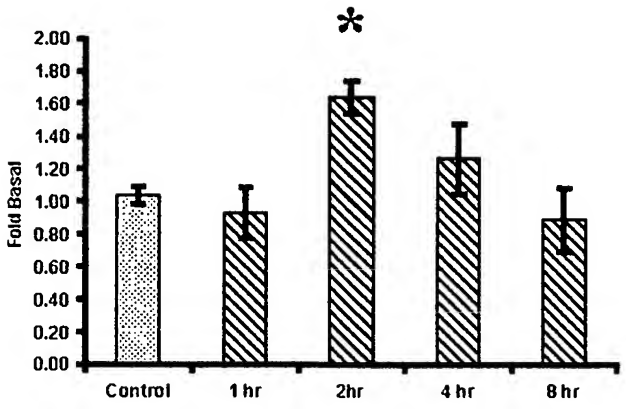

Fig. 3. Time-course of DEXNOR-induced changes in APP_s levels. Animals received a single intraperitoneal injection of DEXNOR (1 mg/kg), and CSF was collected 2 h later. CSF APP_s levels peaked (*$P<0.05$) at 2 h and gradually decreased thereafter. Values represent mean±S.E.M.

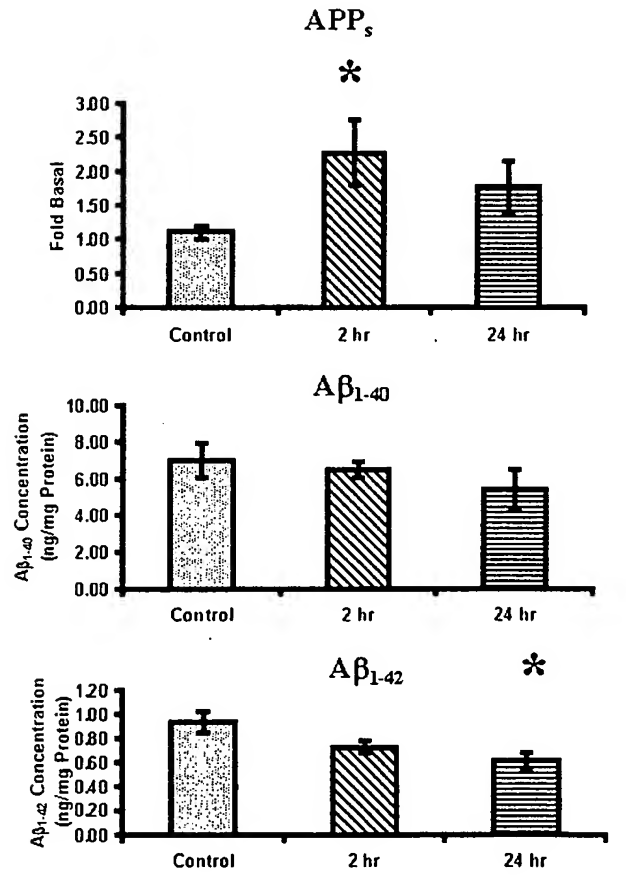

Fig. 4. Effect of chronic administration of DEXNOR (1 mg/kg, bid) on CSF levels of APP_s, $A\beta_{1-40}$ and $A\beta_{1-42}$. Samples were collected 2 and 24 h after DEXNOR administration. APP_s levels were increased (*$P<0.05$) 2 and 24 h after the last injection, while levels of $A\beta_{1-42}$ were decreased (*$P<0.05$) after 24 h. However, $A\beta_{1-40}$ levels were not significantly decreased by DEXNOR administration. Values represent mean±S.E.M.

following a single intraperitoneal injection of DEXNOR, APP_s levels peaked after 2 h and gradually decreased thereafter (0.929±0.15-, 1.64±0.103-, 1.26±0.216- and 0.890±0.197-fold, respectively) (Fig. 3).

Chronic administration of DEXNOR (1 mg/kg) significantly ($P<0.05$) increased CSF APP_s levels, relative to those of controls, 2 h following the last injection of DEXNOR (2.27±0.48-fold) but not 24 h after that injection (Fig. 4). CSF $A\beta_{1-42}$ levels were significantly decreased ($P<0.05$) by chronic DEXNOR treatment at both 2 and 24 h following the last injection of DEXNOR (0.72±0.05 and 0.61±0.07 ng/mg protein, respectively) (Fig. 4). A decrease in CSF $A\beta_{1-40}$ levels occurred 24 h following DEXNOR administration. This decrease, however, was not statistically significant.

Neither hippocampal nor cortical APP_h levels (Fig. 5), nor body weight (Fig. 6), were significantly affected by chronic DEXNOR treatment at the single dosage (1 mg/kg) tested (Fig. 5). GFAP levels in cortex or hippocampus also were unaffected by chronic DEXNOR treatment (results not shown), suggesting that this treatment was not neurotoxic. Chronic treatment with mCPP also increased CSF APP_s levels relative to those in controls ($P<0.05$) when measured 2 h after the last dose (1.81±0.28-fold basal) (Fig. 7), but failed to affect hippocampal or cortical APP_h levels (1.03±0.07- and 1.05±0.11-fold basal, respectively) (Fig. 7B). Unlike DEXNOR, mCPP administration decreased ($P<0.05$) body weight gain (Fig. 7C).

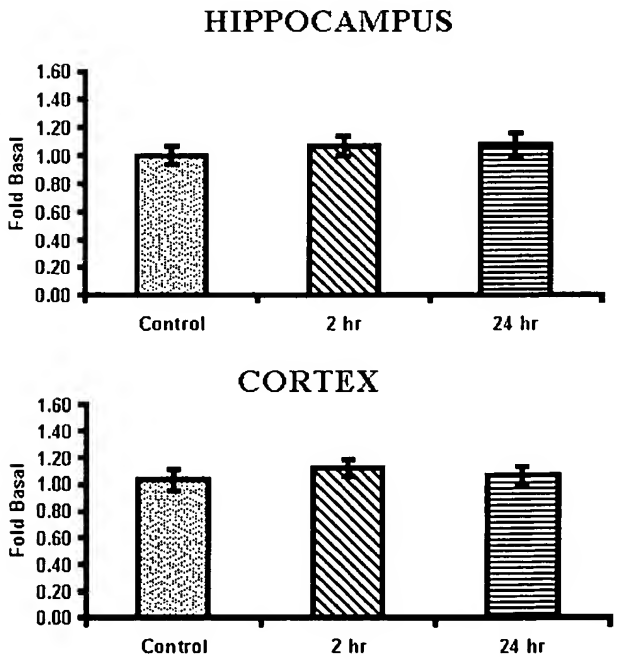

Fig. 5. Hippocampal and cortical APP_h levels in guinea pigs which received DEXNOR chronically, as described in Fig. 4. No significant changes were observed. Values represent mean±S.E.M.

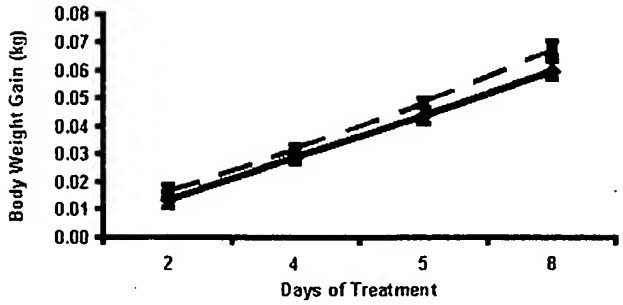

Fig. 6. Body weight of guinea pigs injected with DEXNOR chronically, as described in Fig. 4. No significant changes were observed on body weight. Treated (hatched); control (solid). Values represent mean±S.E.M.

4. Discussion

These data demonstrate that DEXNOR administration affects brain APP metabolism in vivo, increasing CSF APP_s levels (Figs. 1 and 4), probably by activating 5-HT_{2C} receptors. Although CSF APP_s levels increased 2 h after a single dose of the drug, CSF $A\beta_{1-42}$ levels failed to change at this time. However, chronic treatment with DEXNOR (1 mg/kg, bid) for 9 consecutive days increased CSF APP_s levels and concurrently decreased those of $A\beta_{1-42}$ (Fig. 4). These changes were not associated with changes in body weight (Fig. 4), nor with neurochemical evidence of neurotoxicity (i.e. elevated GFAP).

The rapid (i.e. within 2 h) increase in CSF APP_s levels caused by DEXNOR most likely is mediated by the same mechanism as that previously shown to operate in 3T3 cells [17], namely, activation of phospholipase C, metabolism of membrane phospholinositol (PI) to yield DAG, which activates PKC, and PKC-related activation of α-secretase activity. That the rise in CSF APP_s was not caused by enhanced APP_h synthesis is suggested by the speed of this response, and by the failure of chronic DEXNOR treatment to affect hippocampal or cortical APP_h levels (Fig. 5).

In vitro, agonists that stimulate intracellular cAMP formation can cause APP overexpression [10,11], while those like DEXNOR which are coupled to PI hydrolysis do not have this effect [16]. Cultured rat astrocytes treated with DEXNOR increased both PI hydrolysis and APP_s secretion, but did not increase cAMP, APP mRNA nor APP_h levels. In contrast, treatment of cultured rat astrocytes with prostaglandin E2 or norepinephrine induced the formation of cAMP, APP mRNA, and APP_h and of GFAP, a marker for astrocytic activation. DEXNOR also failed to increase GFAP or APP_h levels in the present study, suggesting that DEXNOR is not coupled to cAMP formation in vivo.

Besides activating 5-HT_{2A} and 5-HT_{2C} receptors, DEXNOR also enhances serotonin release and is a potent inhibitor of serotonin re-uptake [4,13]. DEXNOR is the

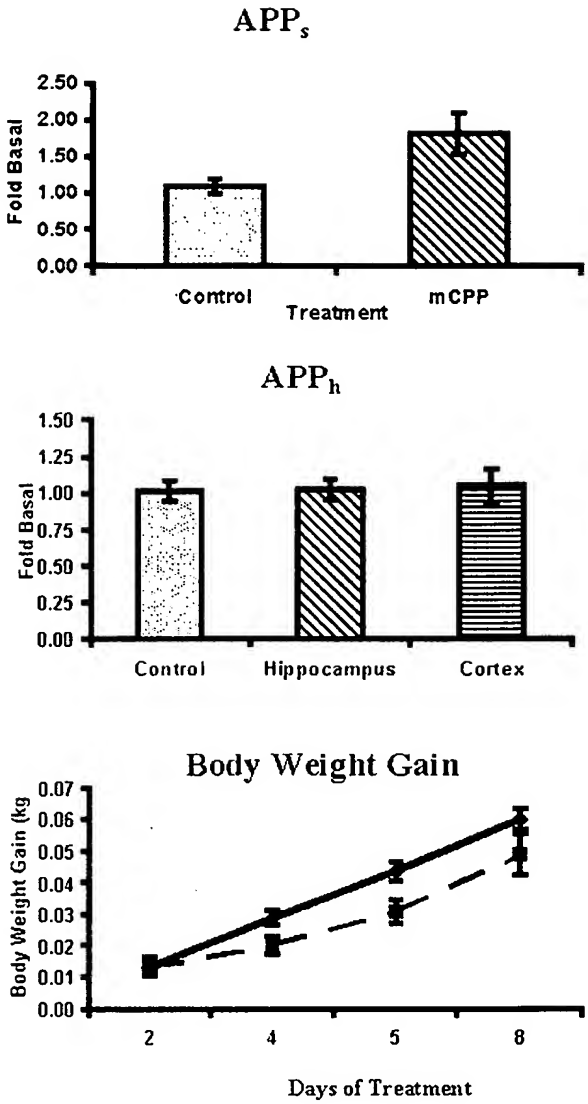

Fig. 7. Effect of chronic administration of mCPP (2 mg/kg, bid) on CSF APP_s, cortical and hippocampal APP levels, and body weight gain. The mCPP treatment increased (*$P<0.05$) CSF APP_s levels relative to controls when measured 2 h after administration (1.81±0.28-fold basal). Neither hippocampal nor cortical APP_h levels were significantly altered by chronic mCPP treatment. mCPP caused a significant (*$P<0.05$) decrease in body weight gain. Treated (hatched); control (solid). Values represent mean±S.E.M.

major metabolite of dexfenfluramine, and the activation of 5-HT-2 receptors that follows dexnorfenfluramine administration mediates dexfenfluramine's anorexic effects [23].

In 3T3 cells transfected with 5-HT_{2A} or 5-HT_{2C} receptors, the stimulatory effect of DEXNOR on APP_s secretion was inhibited by the serotonin antagonists ritanserin or ketanserin [17]. In our in vivo study, the stimulatory effect of DEXNOR on CSF APP_s was completely inhibited by ritanserin, a 5-$HT_{2A/2C}$ antagonist, but not affected by ketanserin, a 5-HT_{2A} antagonist (Fig. 2).

Furthermore, chronic administration (2 mg/kg bid) of mCPP, a 5-HT $_{2B/2C}$ receptor agonist that is structurally unrelated to DEXNOR [20], caused similar increases in CSF APP_s (Fig. 7). Chronic administration of mCPP at this dosage, however, caused a significant reduction in body weight. These results suggest that, in vivo, the main effect of DEXNOR is mediated via 5-HT_{2C} receptors.

APP overexpression and Aβ production are potentially neurotoxic, while APP_s has neurotrophic and neuroprotective functions in vitro [12,21]. In rats, infusion of APP_s improved cognition and synaptic density [22] and enhanced memory retention in a variety of learning tasks [14]. Our data indicate that serotoninergic compounds like DEXNOR that activate brain 5-HT_{2C} receptors may be useful for treating AD by increasing APP_s formation and decreasing that of $A\beta_{1-42}$. Because 5-HT_{2C} receptors apparently are localized to the brain [7,15,19] 5-HT_{2C} agonists may not produce peripheral side effects as seen with other drugs (e.g. muscarinic receptor agonists) that similarly promote non-amyloidogenic APP cleavage.

Acknowledgements

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